APPENDIX D

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Mutations at the arginine residues in α8 loop of *Bacillus thuringiensis* δ-endotoxin Cry1Ac affect toxicity and binding to *Manduca sexta* and *Lymantria dispar* aminopeptidase N

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Abstract The functional role of the α8 loop residues in domain II of *Bacillus thuringiensis* Cry1Ac toxin was examined. Alanine substitution mutations were introduced in the residues from 275 to 293. Among the mutant toxins, substitutions at R281 and R289 affected toxicity to *Manduca sexta* and *Lymantria dispar*. Loss of toxicity by these mutant toxins was well correlated with reductions in binding affinity for brush border membrane vesicles and the purified receptor, aminopeptidase N (APN), from both insects. These data suggest that the two arginine residues in the α8 loop region are important in toxicity and APN binding in *L. dispar* and *M. sexta*.

Key words: Brush border membrane vesicle; Aminopeptidase N; Surface plasmon resonance; *Bacillus thuringiensis*; *Lymantria dispar*; *Manduca sexta*

1. Introduction

Despite its importance as a systemic pesticide in genetically modified plants, the mechanism of action of *Bacillus thuringiensis* (Bt) δ-endotoxin remains unclear. It is generally accepted that Bt crystals are ingested by susceptible insect larvae, solubilized in the midgut, and simultaneously digested to active toxins by midgut proteases. Activated toxins bind to specific receptor molecules present in the midgut epithelial cell brush border membrane and insert into the membrane, forming a pore or ion channel that ultimately kill the insect. The details of how Bt toxins associate with receptors and insert into membranes is still an area of active research and controversy.

Solution of the X-ray crystal structures of Cry3A and Cry1Aa revealed a conserved three-domain composition of Bt toxins [1,2]. Site-directed mutagenesis has been conducted extensively within each domain to localize regions responsible for toxicity, receptor binding and pore formation. Briefly, domain I contains seven α-helices and is believed to be responsible for membrane spanning and pore formation. Site-directed mutagenesis in the helices altered toxicity by changing pore-forming activity. Domains II and III are composed almost exclusively of β-sheets and turns. Domain II was originally proposed to be a receptor-binding domain. Mutations in the loop regions (α8, loop 1, 2, 3) of domain II affected toxicity by changing reversible or irreversible BBMV binding. Domain switches and site-directed mutagenesis of domain III demonstrated that domain III is important in specificity, receptor binding, and pore formation [3].

In order to understand toxicity, specificity, and in some cases, insect resistance mechanisms, the receptor-binding properties of Bt toxins have been extensively examined using brush border membrane vesicles (BBMV) [4–6]. To directly examine toxin receptor-binding properties, several Bt toxin receptors have been identified. A Cry1A toxin-binding protein, 120-kDa aminopeptidase N, has been identified and purified from *Manduca sexta*, *Lymantria dispar*, and other insects and its gene was cloned [7–11]. A 210-kDa cadherin-like molecule was also identified as a Cry1A toxin receptor [12]. Recently, interaction between Bt toxin and purified receptor has been studied using surface plasmon resonance (SPR) [13–17]. To *M. sexta* APN, Cry1Ac binds to two sites with 2:1 toxin receptor stoichiometry. One site requires only domain III, whereas the other requires both domain III and domain II for complete binding [16,18]. However, it has been demonstrated that domain III binding of *M. sexta* APN does not correlate with toxicity [19–21], but domain II binding does [22]. There is evidence that domain II of Cry1Ac is necessary for binding not only APN, but also the 210-kDa receptor [16].

In *L. dispar*, Cry1Ac binds APN with a 1:1 stoichiometry. A recent study on the binding of Cry1Ac to the *L. dispar* APN proposed that a cavity in lectin-like domain III initiates docking through recognition of an *N*-acetylgalactosamine moiety on APN, then a higher affinity binding occurs through domain II [17]. Like *M. sexta*, it was observed that domain II mutations had a profound effect on insecticidal activity compared to domain III mutations. These findings were further supported by mutations at R368 and R369 of Cry1Ac domain II, which dramatically affected toxicity, BBMV binding, and APN binding in *M. sexta* and *L. dispar* [22].

A previous study showed that the mutations in the α8 loop of domain II of Cry1Ab affected toxicity and BBMV binding to *L. dispar*. Decreased activity was directly correlated with the reduced binding affinity for BBMV [23]. In the present

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study, the functional role of α8 loop of Cry1Ac toxin is examined. Alanine substitutions were made at the residues from 275 to 293 and the biological activities against *M. sexta* and *L. dispar* were determined. BBMV-binding assays and SPR-binding assays with aminopeptidase N purified from both insects were also performed. Two arginine residues in the α8 loop region were found to be important in binding to BBMV and APN as well as insecticidal activity against both lepidopteran species.

2. Materials and methods

2.1. Cry1Ac mutant construction and toxin purification

The *cry1Ac1* gene (pOS4201) was subcloned into pBluescript KS+ (pOS11200) and expressed in *Escherichia coli* MV 1190. Cry1Ac mutants were constructed as described previously [24]. Inclusion bodies were purified and solubilized in 50 mM sodium carbonate buffer, pH 9.8, containing 10 mM DTT. The solubilized protoxin was digested with 2% trypsin (Sigma) at 37°C for 2 h. An additional dose of 1% trypsin was added and further incubated for 2 h. Protein concentration of protoxins and toxins was estimated by Coomassie protein assay reagent (Pierce), and the purity was examined by 10% SDS–PAGE. Toxin was further purified using a size-exclusion Superdex 200 column on an AKTA Explorer (Pharmacia Biotech AB, Uppsala, Sweden). Toxin was eluted with 20 mM phosphate buffer, pH 7.4, at 1 ml/min flow rate, or 10 mM HBS buffer (10 mM HEPES, pH 7.4, 150 mM NaCl and 3.4 mM EDTA) for BIAcore studies. CD spectra analysis of toxins was conducted as previously described [19].

2.2. Insect bioassays

L. dispar eggs were kindly supplied by Gary Bernon (US Department of Agriculture, Otis Methods Development Center, Beltsville, MD, USA). *M. sexta* eggs were kindly supplied by D.L. Dahlman (Dept. of Entomology, University of Kentucky, Lexington, KY, USA). Eggs were hatched and reared on artificial diet (Bio-serv, Frenchtown, NJ, USA). Activities of toxins were determined with 2–3-day-old *L. dispar* and *M. sexta* larvae by the surface contamination method as described [25]. Toxins were diluted in 50 mM sodium carbonate buffer (pH 9.5), and 35-μl samples were applied per well (2 cm^2) on artificial diet in 24-well tissue culture plates. Two larvae were placed in each well and the mortality was recorded after 5 days. Bioassays were repeated at least five times. The effective dose estimates (LC_{50}, 50% lethal concentration of toxin) were calculated using PROBIT analysis [26].

2.3. BBMV-binding assays

BBMV was prepared from the last instar larval midguts of *L. dispar* and *M. sexta* by the magnesium precipitation method [27]. 20 μg of Cry1Ac toxin was iodinated with 1 mCi of $Na^{125}I$ (Dupont) and an IODO-BEAD (Pierce). Labeled toxin was separated from the free iodine using an Excellulose GF-5 column (Pierce). Homologous and heterologous competition binding assays were performed as described previously (MolMicro paper, 2000). K_{com} (nM) and B_{max} (pmol/mg of BBMV) values were calculated with the computer program LIGAND [28]. K_{com} represents the binding affinities calculated from BBMV competition binding experiments. This term is used to reflect the lack of steady state binding kinetics due to the irreversible membrane insertion event [29].

2.4. SPR binding assays

Binding of toxin to aminopeptidase N was studied using BIAcore®2000 with CM5 sensor chips (BIAcore AB, Uppsala, Sweden). APNs were purified from *M. sexta* and *L. dispar* BBMVs using chromatographic methods as previously described [16,17]. APN diluted in 20 mM ammonium acetate buffer, pH 4.1, was immobilized onto the carboxymethylated dextran surface of a CM 5 sensor chip using amine coupling method according to BIAcore standard protocols. All immobilization reagents were provided from the BIAcore coupling kit. Regeneration of receptor was performed with 30 μl of 10 mM NaOH, pH 11 at 100 μl/min. APN was immobilized at three different densities from approximately 75–800 resonance units (RU). One flowcell per chip served as a control surface containing non-toxin-binding APN treated with mild alkaline hydrolysis. A buffer flow rate of 30 μl/min was used for both association and dissociation. Toxins were injected at five different toxin concentrations (100, 200, 500, 750, 1000 nM) in random order. The toxin–receptor complex was allowed to dissociate for at least 5 min after 4 min of injection.

SPR data were analyzed with global fitting in BIAevaluation 3.0. For Cry1Ac binding to *M. sexta* APN, a complex two-binding site (heterogeneous ligand) model (A+B1+B2↔AB1+AB2) was employed. Rationale for fitting to this model has been described [13]. Response curves generated by Cry1Ac binding to *L. dispar* APN were analyzed using the two-state (conformational change) model, as described [17]. Apparent rate constants have standard errors less than 10% of the values reported.

3. Results and discussion

3.1. Expression of mutant toxins and biological activities

The Cry1Ac α8 loop residues mutated in this study are shown in Fig. 1. Alanine substitution mutant toxins, N275A, F276A, D277A, S279A, F280A, R281A, S283A, R289A, S290A, and S293A produced stable protoxins and the yields were comparable to wild-type. After trypsin digestion, all mutant proteins produced stable toxin fragments like wild-type, as determined by SDS–PAGE and CD spectra analysis (data not shown). Biological activities of the mutant toxins against *L. dispar* and *M. sexta* larvae were determined. The mutant toxins, N275A, F276A, D277A, S279A, F280A, S283A, S290A, and S293A exhibited similar toxicity as Cry1Ac toxin to both insects. The LC_{50} values ranged from 2.5 to 5.7 ng/cm^2 to *M. sexta* larvae and 5.9–10.3 ng/cm^2 to *L. dispar* larvae. On the other hand, two arginine mutant toxins, R281A and R289A, showed great reductions in toxicity to both insects (Table 1). To *L. dispar* larvae, R281A and R289A toxins exhibited 100-fold and 49-fold reduced toxicity, respectively. To *M. sexta* larvae, R281A and R289A showed 48-fold and 30-fold reduced toxicity. These data strongly suggest that the arginine residues in this loop are important for toxicity.

3.2. BBMV-binding assays

BBMV competition binding assays were performed with

Table 1
Biological activity and affinity of Cry1Ac mutant toxins to *L. dispar* and *M. sexta*

Insect	Toxin	LC_{50} (ng/cm^2)[a]	Toxicity loss[b]	K_{com} (nM)[c]	K^c_{D1} (nM)	K^c_{D2} (nM)
L. dispar	Cry1Ac	7.5	1	2.3	–	210
	R281A	749	100	34.4	–	850
	R289A	365	49	65.3	–	525
M. sexta	Cry1Ac	5.3	1	5.3	100	77
	R281A	255	48	25.3	165	267
	R289A	157	30	54.5	94	123

[a]50% lethal concentration.
[b]LC_{50}mut/LC_{50}wt.
[c]BBMV-binding affinity.

iodine-labeled Cry1Ac toxin. To *L. dispar* BBMV, wild-type Cry1Ac binds with high affinity, yielding a K_{com} of 2.3 nM. The toxic mutant proteins (N275A, F276A, D277A, S279A, F280A, S283A, S290A, and S293A) bound to BBMV with affinities similar to Cry1Ac (data not shown). However, R281A and R289A mutant toxins bound to BBMV with lower affinities of 34.4 and 65.3 nM, respectively (Fig. 2). To *M. sexta* BBMV, wild-type Cry1Ac binds with a K_{com} of 5.3 nM. The toxic mutant proteins (N275A, F276A, D277A, S279A, F280A, S283A, S290A, and S293A) bound to BBMV like wild-type (data not shown), whereas R281A and R289A mutant toxins bound to BBMV with reduced affinities of 25.3 nM and 54.5 nM, respectively (Fig. 2). BBMV competition binding data are well correlated with toxicity data, in which less toxic proteins (R281A and R289A) bound to BBMVs with lower binding affinities. Dissociation binding assays were also performed to determine whether the loss of toxicity and competition binding were influenced by irreversible binding properties of the mutant toxins. No measurable differences between mutants and wild-type were observed in dissociation binding assays (data not shown). This indicated that the loss of BBMV affinity might be solely due to loss of reversible BBMV binding, presumably caused by a reduced affinity for membrane receptors.

3.3. Kinetic analysis of APN receptor binding by SPR

In order to further examine the molecular basis of the reduction in toxicity and BBMV binding of Cry1Ac mutant toxins, SPR-binding assays were performed with Cry1Ac receptor APN purified from *L. dispar* and *M. sexta*. Previously, much lower binding affinity has been reported for Cry1Ac toxin binding to purified APN using SPR compared to Cry1Ac toxin affinity for BBMV [15]. In contrast, a 210-kDa cadherin-like protein identified as a Cry1Ab receptor

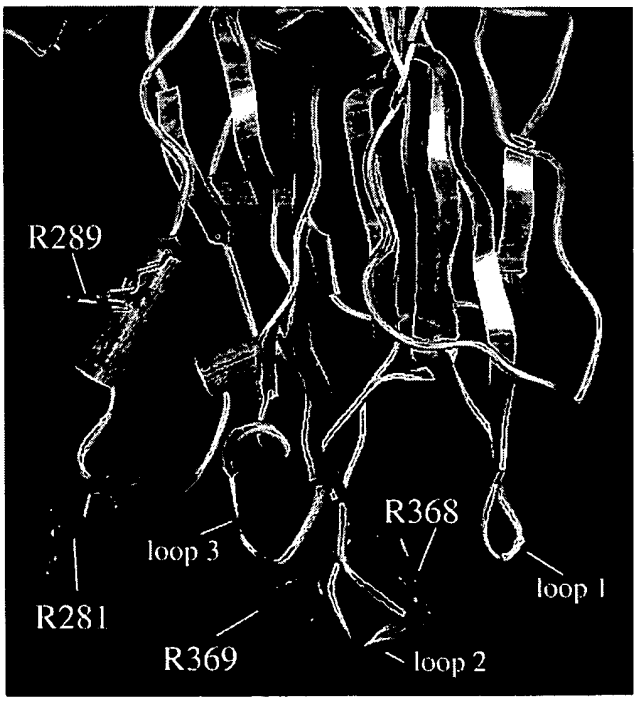

Fig. 1. Ribbon display of the Cry1Ac domain II predicted three-dimensional structure. R281, R289, R368 and R369 residues mutated in this study are shown in stick mode.

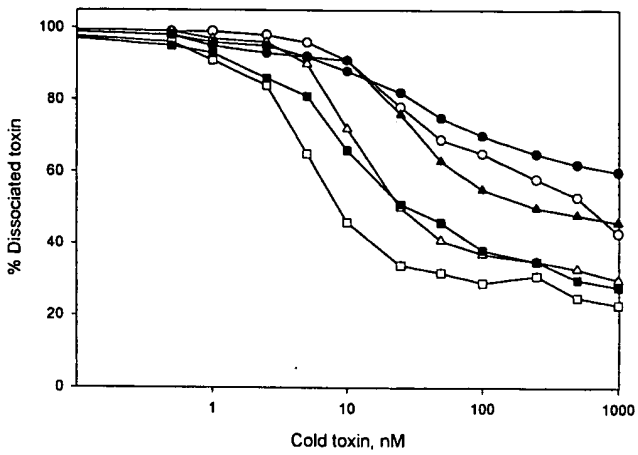

Fig. 2. Binding of Cry1Ac mutant toxins to *L. dispar* BBMV and *M. sexta* BBMV. ^{125}I-labeled Cry1Ac toxin (2 nM) was incubated with 10 μg of BBMV in the presence of increasing concentration of unlabeled Cry1Ac. Binding is expressed as a percentage of the amount bound upon incubation with labeled toxin alone. Open symbols, *M. sexta*; filled symbols, *L. dispar*. Cry1Ac toxin, squares; R289A toxin, triangles; and R281A toxin, circles.

in *M. sexta* showed binding affinities for Cry1Ab toxin similar to BBMV [12], although the value was not obtained by SPR. The question of how many receptors function in toxicity still remains to be answered. Fig. 3A shows the sensorgram of Cry1Ac and mutant toxins, R281A and R289A, binding to *M. sexta* APN. Both R281A and R289A displayed considerable reductions in binding to *M. sexta* APN compared to wild-type Cry1Ac. Fig. 3B shows the sensorgram of Cry1Ac and mutant toxins, R281A and R289A to *L. dispar* APN. The mutant toxins also showed weaker binding to *L. dispar* APN. The reduction in overall binding affinities for *M. sexta* APN appears to derive primarily from one of the two sets of rate constants (K_{D2}, data available on request), where R281A and R289A showed 3.5-fold and 1.6-fold losses in affinity, respectively (Table 1). These data suggest that the α8 residues important for binding and toxicity might be involved in binding to one of the *M. sexta* APN-binding sites. Alterations in both the rates of complex association (k_{a2}) and dissociation (k_{d2}) were obtained (data not shown). Similarly, the reduction in overall K_D for the mutant binding to *L. dispar* APN was due to alterations in k_{a2} and k_{d2} apparent rate constants. R281A and R289A showed 4-fold and 2.5-fold losses in affinity to *L. dispar* APN overall. The correlation of our mutants' biological activity and APN binding further supports a functional role for APN in Bt toxicity to lepidopterans.

In this study, novel α8 loop mutations in domain II of Cry1Ac were shown to affect binding to BBMV and purified APN receptor, which diminished insecticidal activity toward the lepidopteran pests *L. dispar* and *M. sexta*.

Although the α8 mutations in this study occur in domain II of Cry1Ac, recent SPR studies employing mutagenesis of domain III or domain swapping have shown that domain III is critical for Cry1Ac binding to purified APN from either *L. dispar* or *M. sexta* [16,17]. Further, the contact of Cry1Ac with *M. sexta* and *L. dispar* APN appears to be initiated by docking of an *N*-acetylgalactosamine moiety on APN in a Cry1Ac domain III cavity [17,20,21]. Despite the importance of domain III for APN binding, no direct correlation with toxicity has been observed for either *L. dispar* or *M. sexta*.

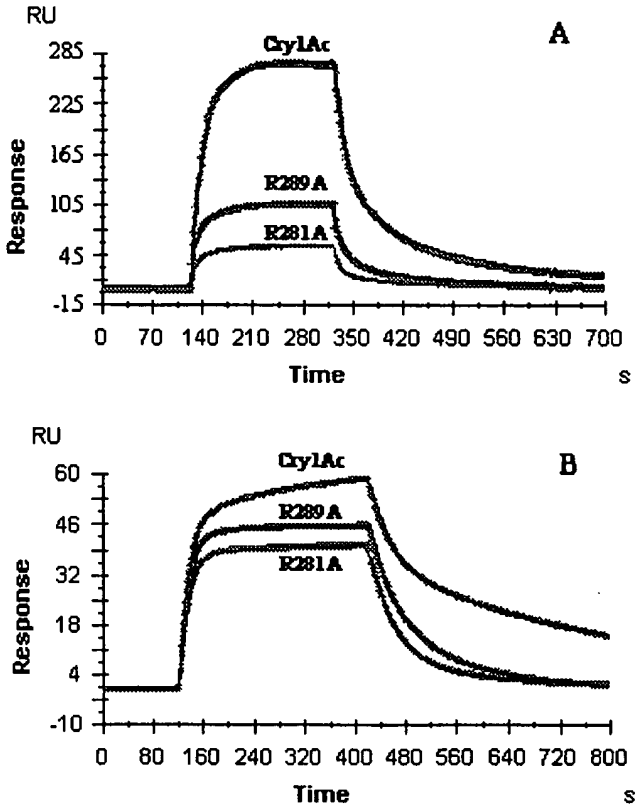

Fig. 3. Binding of Cry1Ac and mutant toxins to immobilized lepidopteran APNs. Toxins diluted in HBS buffer were injected over immobilized APN at 30 μl/min, 25°C. For dissociation, toxin flow was replaced by HBS buffer and recorded for at least 300 s. Experimental curves are gray and simulated fitted curves are overlaid in black. A: A representative binding curve overlay of Cry1Ac and mutant toxins (250 nM) injected over *M. sexta* APN (300 RUs) for 240 s. B: A representative binding curve overlay of Cry1Ac and mutant toxins (250 nM) injected over *L. dispar* APN (150 RUs) for 300 s.

Although domain III mutant toxins (Q509A, R511A, 509QNR-AAA511) eliminated APN binding, toxicity decreased no more than 2-fold, suggesting an alternative receptor for Cry1Ac is present in BBMV from both these insects. Therefore, analysis of toxin binding by domain III mutants to APN is insufficient for predicting toxicity [17,19,21]. On the other hand, R281A and R289A, as well as several other domain II loop mutations, not only affect APN binding but retain strong correlation between toxicity and binding affinity [17]. Despite this correlation, the loss in BBMV binding and toxicity to *L. dispar* and *M. sexta* by R281A and R289A mutant toxins does not appear to be accounted for entirely by loss of APN binding. Previously, ligand blotting experiments have been used to localize the receptor-binding domain using domain-switched hybrid toxins. These studies provided evidence that domain II of Cry1Ac might be involved in binding the 210-kDa *M. sexta* receptor [18]. It is likely that domain II mutations, such as R281A and R289A, affect binding to other functional receptors in addition to APN, allowing binding and toxicity to better correlate than domain III mutant toxins. Alternatively, domain II may play a greater role in toxicity beyond receptor recognition, such as facilitating a conformational change for membrane insertion.

Arginine substitutions similar to those in this report have been introduced in the α8 loop of Cry1Aa and the mutant toxins were tested to both insects. R281A mutant toxin was not stable by trypsin digestion. Only R286A mutant toxin greatly affected toxicity and BBMV binding to *L. dispar* (M.K. Lee and D. Dean, unpublished). From these studies, arginine residues in the α8 loop seemed to be involved in initial recognition of receptors for Cry1Aa as well. Another study with Cry1Ab and Cry1Ac double arginine mutant toxins (R368/R369) also supports that the positive charges are involved in the initial recognition to the receptor on BBMV [17,22]. Pertinent to this discussion is the observation that the pH of the alimentary canal of various lepidopteran species ranges from 9 to 11.5, depending on the position in the midgut [30]. Since the p*K* of the arginine guanidino group is above 12, this may represent an important long-range force involved in orienting the correct toxin-binding position on receptors with a presumably negative surface. Overall, positive charges may play a conserved role in Cry toxin affinity for receptors in lepidopteran midguts.

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